Mode of Action and Bactericidal Properties of Surotomycin against Growing and Non-growing *Clostridium difficile*

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ABSTRACT

Surotomycin (CB-183,315), a cyclic lipopeptide is in phase 3 clinical development for the treatment of *Clostridium difficile* infection. We report further characterization of the *in vitro* mode of action of surotomycin, including its activity against growing and non-growing *C. difficile*. This was assessed through time kill kinetics, determining effects on the membrane potential and permeability and macromolecular synthesis in *C. difficile*. Against representative strains of *C. difficile*, surotomycin displayed concentration-dependent killing of both logarithmic-phase and stationary-phase cultures at a concentration that was ≤16-fold more than the minimum inhibitory concentration (MIC). Exposure resulted in the inhibition of macromolecular synthesis (DNA, RNA, protein and cell wall). At bactericidal concentrations, surotomycin dissipated the membrane potential of *C. difficile* without changes to the permeability of propidium iodide. These observations are consistent with surotomycin acting as a membrane-active antibiotic, exhibiting rapid bactericidal activities against growing and non-growing *C. difficile*.

INTRODUCTION

The Gram-positive, spore-forming anaerobic bacterium *Clostridium difficile* is the leading cause of hospital-acquired diarrhea in North America and Europe (1, 2). Elderly hospitalized patients on broad-spectrum antibiotics are the main target populations, but recent observations indicate there is an increase in the incidence of *C. difficile* infection (CDI) in the community without known risk factors (3, 4). In the United States in 2011 there were an estimated 500,000 cases of CDI resulting in 29,300 deaths (5), reflecting the devastating impact of CDI since the turn of the last century. Furthermore, the number of cases of severe CDI has escalated, coinciding with the emergence of epidemic ribotypes such as BI/NAP1/027 (2, 6). BI/NAP1/027 is now responsible for a significant number of cases of hospital-acquired CDI in North America (5, 6).

For more than 30 years vancomycin and metronidazole have been the first-line treatment choices for CDI (7). Metronidazole is prescribed for mild to moderate CDI, while vancomycin is recommended for severe CDI (6, 8). However, rates of recurrence of 20-25% or higher in severe CDI are common, following treatment with metronidazole or vancomycin (6, 9, 10). The mode of action of vancomycin is well established, involving inhibition of the latter stages of peptidoglycan biosynthesis, which primarily kills rapidly growing *C. difficile* (11). Metronidazole undergoes biochemical reduction to form reactive species that target DNA and is potent in vitro, but only low concentrations reside in the gastrointestinal tract (12-16). Fidaxomicin, which targets the bacterial RNA polymerase inhibitor, has a narrower spectrum of activity than metronidazole and vancomycin and is superior in the prevention of CDI recurrence (17, 18). However,

additional novel therapeutics are required to effectively treat CDI and reduce the rates of recurrence following initial therapy.

Surotomycin is a minimally absorbed narrow-spectrum cyclic lipopeptide antibiotic, which is in phase 3 clinical trials as a novel treatment for CDI. It is chemically and structurally related to the antibiotic daptomycin that targets the bacterial membrane thereby exhibiting bactericidal effects (19-21). Daptomycin has been shown to display activities against stationary phase *Staphylococcus aureus* (21), which is a property that would seem amenable to the action of surotomycin in mitigating the pathogenesis of *C. difficile*. This organism produces spores and toxin (TcdA and TcdB), primarily in the late logarithmic and stationary phases of growth (22). However, it is unreported whether the bactericidal activity of surotomycin encompasses the non-growing stationary phase *C. difficile*. Killing of stationary phase cells by membrane-active antibiotics has been shown to lower toxin and spore numbers in vitro, which in principle could contribute to lowering disease severity and rates of endogenous recurrence (11). The basis for surotomycin's potent activity against *C. difficile* is thought to arise from dissipation of the bacterial membrane as shown in *S. aureus* (19). However, direct studies determining if surotomycin dissipates the membrane potential of *C. difficile* have not been reported. Herein, we characterized the mode of action of surotomycin against *C. difficile*, examining its bactericidal effects on logarithmic and stationary phase cells and associated cellular effects linked to dissipation of the membrane potential in *C. difficile*. This study was presented in part as a poster presentation at the 54th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC).

MATERIALS AND METHODS

Compounds, bacterial strains, and growth media. Surotomycin and daptomycin were provided by Merck and Co., Inc. All other antimicrobials were obtained from Sigma-Aldrich (vancomycin, metronidazole, CCCP, ampicillin, fusidic acid, rifaximin and nisin) or Enzo Life Sciences (gatifloxacin). The *C. difficile* strains BAA-1875 (ribotype 078) and BAA-1803 (ribotype 027) were from the American Type Culture Collection (ATCC). Strain R20291 (ribotype 027) was kindly provided by Dr. A. L. Sonenshein, Tuft University, Boston USA. Strain IT0843 (ribotype 001) was kindly provided by Dr. Paola Mastrantonio (Istituto Superiore di Sanità, Rome, Italy). Brain Heart Infusion (BHI; from Oxoid) was used for all the experiments and was supplemented with calcium to a final concentration of 50 mg/L for all experiments with surotomycin and daptomycin. To supplement BHI to a final calcium concentration of 50 mg/L, calcium levels in manufactured lots of BHI were determined by the Laboratory Specialists, Inc., OH. All strains were routinely grown in pre-reduced BHI media under anaerobic conditions in a Whitley A35 anaerobic workstation at 37°C.

Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs). Minimum inhibitory and minimum bactericidal concentrations of compounds against *C. difficile* were determined as described by Wu et al. (11, 23). MICs were performed using ~10^6 cfu/mL inoculum of *C. difficile* in 24-well microtitre plates containing 2-fold serial dilutions of compounds in a total volume of 1 mL. MICs were defined as the lowest concentrations of compounds inhibiting visible growth after 24 h of incubation. MBCs were performed against both the logarithmic-

phase (MBC_{Log}) and stationary-phase (MBC_{Sta}) cells using logarithmic (OD_{600nm}≈0.3) and 24 h old cultures, respectively. Briefly, in 24-well microtitre plates, cultures were added to 2-fold diluted compounds in a total volume of 1 mL. After 24 h of incubation, the number of viable cells was determined by plating aliquots onto pre-reduced BHI agar containing activated charcoal (10 % w/v). MBCs were defined as the lowest concentrations of compounds causing ≥3 log reduction in viable cells compared to the starting inocula. The MICs and MBCs were determined from two independent starting cultures.

Time-kill kinetics, Were evaluated against both the logarithmic and stationary phase cultures as described by Wu et al. (11, 23). Logarithmic (OD_{600nm} ≈0.3) and stationary-phase cultures were exposed to 1, 4 and 16× the MIC of compounds. Samples (100 µL) were taken at time 0, 1, 2, 4, 6 and 24 h after the addition of compounds and viable cell counts were determined on BHI agar plates containing activated charcoal (10 % w/v). Bacterial counts were enumerated after 24 h of incubation. This assay were determined from two independent starting cultures.

Effects on macromolecular biosynthesis. Logarithmic cultures of R20291 and BAA-1875 were grown to early logarithmic-phase (OD_{600nm} ≈0.3) under anaerobic conditions and aliquoted for subsequent analysis. To analyze the DNA, RNA, protein and cell wall synthesis inhibition; ^{3}H-Thymidine (2 µCi/mL), ^{3}H-Uridine (2 µCi/mL), ^{3}H-Threonine (2 µCi/mL) and ^{3}H- N-acetyl-Glucosamine (2 µCi/mL) were used respectively. Radiolabelled precursors were added 5 min before the addition of compounds at either

131 inhibitory (1×MIC) and bactericidal (16×MIC) concentrations. Gatifloxacin, rifaximin,
132 fusidic acid and ampicillin were used as controls for DNA, RNA, Protein and cell wall
133 synthesis inhibition, respectively. Against BAA-1875 strain, gatifloxacin, rifaximin,
134 fusidic acid, and ampicillin concentrations at 16×MIC, were 64 µg/mL, 0.96 µg/mL, 2
135 µg/mL, and 8 µg/mL, respectively. Against R20291, the concentrations at 16×MIC were
136 512 µg/mL, 2 µg/mL, 2 µg/mL, and 16 µg/mL, for the respective control drugs. Samples
137 (500 µL) were taken at specific time points (30, 60 and 120 min), spun down, and the cell
138 pellet collected and incubated on ice with 10% w/v ice-cold trichloroacetic acid (TCA)
139 for 30 min. Samples were then filtered through Whatman GF/C filters, washed twice with
140 5% w/v TCA and 95% ethanol. Filters were dried and scintillation counting performed.
141
142 **Determination of the membrane potential and permeability using FACS.** To assess
143 the effects of compounds on the membrane potential and permeability of *C. difficile*, we
144 adopted a fluorocytometric method relying on the use of $DiBAC_4(3)$ to assess the
145 membrane potential and propidium iodide (PI) to assess membrane permeability. This
146 method was based on that reported by Nuding et al. (24) for anaerobic bacteria;
147 Diethyloxacarbocyanine iodide $DiOC_2(3)$ was found to be inconsistent (*data not shown*).
148 Strains R20291 and BAA-1875 were used, and were exposed to different concentrations
149 of compounds as: 1, 4 and 16× the MIC of compounds. Briefly, cultures were grown
150 anaerobically to an OD_{600nm} ≈0.2 and 10 mL aliquots added to 20 mL serum vials.
151 Compounds were subsequently added and the vials crimped sealed with silicone bungs
152 and removed from the anaerobic chamber. After 10 min of adding compound, $DiBAC_4(3)$
153 was added via a 23G syringe needle to a final concentration of 5 µM. After an overall 30

min of exposing cells to compounds, at room temperature, fluorocytometric analysis was performed using BD LSR II flow cytometer. $DiBAC_4(3)$ was excited using the 488-nm excitation laser and its fluorescence emission detected using FITC filters. As a positive carbonyl cyanide m-chlorophenyl hydrazone (CCCP; Sigma-Aldrich), which completely dissipates the membrane potential was used; vancomycin was used as a negative control; a minimum of three independent cultures was evaluated.

Membrane permeability assays were similarly performed using the protocol described above, except that the membrane impermanent dye PI was added to a final concentration of 5 µM instead of $DiBAC_4(3)$. After 30 min samples were analyzed in the BD LSR II flow cytometer, with excitation at 488-nm and emission collected using the PI-A filters. Nisin was used as a positive control for membrane damage and vancomycin as a negative control. Daptomycin was also included as a control in these experiments. Resazurin (0.001 gm/L) and sodium thioglycollate (0.5 gm/L) were added to the media to act as an indicator of oxygenation and an oxygen scavenger, respectively. Histogram plots of number of events against fluorescence of the population were comparatively analyzed using FlowJo X 10.0.7.

RESULTS

Surotomycin is bactericidal against both the logarithmic and stationary-phase *C. difficile*. As shown in Table 1, surotomycin MICs against test strains ranged from 0.125 to 1 µg/mL. The concentration of surotomycin required for bactericidal activities against logarithmic and stationary phase cultures were similar and was 8-128 fold above the MICs (Table 1), which corresponds to 2 to 16 µg/mL. As expected the control

metronidazole was also bactericidal, killing both culture types at concentrations between 2 to 16 µg/mL, whereas vancomycin was bacteriostatic and was completely inactive against stationary-phase *C. difficile*.

Surotomycin kills *C. difficile* in a concentration-dependent manner. Time kill kinetics were subsequently performed to determine how rapidly surotomycin killed both logarithmic and stationary phase cells. These assays revealed that surotomycin exhibited a concentration dependent mode of killing against both the logarithmic- and stationary-phase cultures of *C. difficile*. Against logarithmic BAA-1875, at 16×MIC (4 µg/mL), surotomycin killed more than 99% of cells in 6 h, whereas 24 h was required to achieve a similar reduction in culture viability, against stationary-phase cells (**Figure 1**). A similar pattern of killing was observed against R20291, although, this strain seemed more sensitive at 4×MIC of surotomycin, since a 99% reduction in viable numbers was observed after 24 h against logarithmic-phase cultures (**Figure 2**). This suggests that against R20291 in larger culture volume (10 mL) the MBC is 4 µg/mL, which differs by 4-fold from that obtained in 1 mL volumes for MBC determinations. Metronidazole was also found to display concentration dependent killing (against both logarithmic and stationary-phase cells), causing a ≥99% reduction of viable cells at 16×MIC after 24 h; whereas vancomycin demonstrated bacteriostatic effect and was completely inactive against stationary-phase cultures. These observations broadly support the above findings of the MBCs data.

Surotomycin dissipates membrane potential without pore formation. In order to examine whether surotomycin dissipates the membrane potential of *C. difficile*, we adopted the fluorescent probe $DiBAC_4(3)$. The fluorescence of $DiBAC_4(3)$ changes with the membrane potential status of cells, with depolarized cells demonstrating enhanced fluorescence, due to $DiBAC_4(3)$ entering depolarized membranes and binding to lipid-rich intracellular components, thereby exhibiting increased green fluorescence (24). When *C. difficile* cultures of R20291 and BAA-1875 were treated with surotomycin or daptomycin, increases in florescence were only observed 16× their MICs, compared to the untreated cultures (**Figure 3**). These concentrations were bactericidal for both agents. No changes in the fluorescence of cells were observed at lower concentrations of surotomycin or daptomycin (i.e. 1 and 4×MIC). As expected, the negative control vancomycin that inhibits peptidoglycan biosynthesis did not alter the membrane potential, while CCCP that acts as a proton ionophore and disrupts the bacterial membrane potential was shown to increase the fluorescence of R20291 and BAA-1875. At the 30 min of treatment, dissipation of the membrane potential by surotomycin and daptomycin at 16× their MICs did not result in membrane pore formation, as cells did not show an increase in propidium iodide fluorescence compared to the untreated control and vancomycin-treated cultures. In contrast, the pore-forming agent nisin caused membrane pore formation, which was evident by an increase in the red fluorescence of cells (**Figure 3**).

As the above observations are based on 30 min incubation periods, we extended out incubation times. Incubation of cultures with 1 and 4×MIC of surotomycin or daptomycin for up to 2 h did not lead to an observable difference in the membrane

potential status of cells compared to untreated controls (*data not shown*). Similarly, continued exposure to 16×MIC of these drugs did not produce further, measurable increases in the dissipation of the membrane potential; extended incubation times at 1, 4, and 16×MIC did not lead to increases in the permeability of cultures to propidium iodide (*data not shown*).

Surotomycin inhibits multiple macromolecular biosynthetic processes. Exposure of R20291 and BAA-1875 to inhibitory (MIC) and bactericidal (16×MIC) concentrations of surotomycin resulted in the simultaneous inhibition of DNA, RNA, Protein and Cell Wall (**Figure 4**). While it is expected that at the bactericidal concentration, all macromolecular processes would be affected in dying cells, these processes were also affected in cells exposed to inhibitory concentrations. This is consistent with the membrane being the primary target for surotomycin action, thereby imposing multiple cellular effects on processes that require membrane homeostasis (25).

DISCUSSION

Recent studies established that the membrane potential of *C. difficile* is critical to the survival of logarithmic and stationary phase cells, making the clostridial membrane an attractive target for agents to treat CDI (11). Dissipation of the membrane potential, resulting in loss of viability in both growing and non-growing cell types has direct relevance to *C. difficile* pathogenesis, as this organism produces its toxins and spores in the late-logarithmic and stationary phases of growth (22). The cyclic lipopeptide drug surotomycin represents the leading example of a membrane-active antibiotic for treating

CDI. In studies herein we validated that surotomycin dissipates the membrane potential of *C. difficile* and this was not associated with the formation of pores at bactericidal concentrations in two test strains. This observation is consistent with a prior report by Mascio et al. (19) where surotomycin dissipated the membrane potential of *S. aureus* without causing pore formation. Dissipation of the membrane potential of *C. difficile* was evaluated using the fluoroprobe $DiBAC_4(3)$ that other studies have adopted to measure the membrane potential in anaerobes and as it appears more reliable than DiOC for anaerobes (24). However, measurable disruptions of the membrane potential in *C. difficile* were only observed at bactericidal concentrations. This might reflect that the magnitude of the membrane potential in clostridia is low (26). Hence the marginal lowering of the membrane potential of *C. difficile* upon exposure to inhibitory concentrations of drug may be challenging to measure using $DiBAC_4(3)$ and other techniques may be required for lower concentrations.

The action of surotomycin against various strains of *C. difficile* resulted in bactericidal activities against logarithmic and stationary phase cultures and imposed multiple cellular effects as evident by widespread disruption of macromolecular processes. In contrast, the cell wall synthesis inhibitor vancomycin was poorly active or bacteriostatic against logarithmic cells and inactive against stationary phase cultures. Metronidazole did reduce the viability of both cell types, but only low concentrations of drug occur in the gastrointestinal tract as the drug is almost completely absorbed following oral administration (27).

The concentration of surotomycin that was required to inactivate both logarithmic and stationary phase cultures was typically 2-16 µg/mL. These levels are well within the

local concentrations of surotomycin (>1,000 µg/g) that is present in the colon of patients following oral administration (data on file at Merck and Co., Inc. Kenilworth, NJ, USA). It is therefore plausible that in the colon surotomycin is bactericidal against both logarithmic and stationary phase cells. This property could reduce both toxin and spore production in vegetative populations, as recently reported in the in vitro human gut model (28). In Phase II clinical trials, lower rates of recurrence was associated with surotomycin treatment compared to oral vancomycin; as 27.9% and 17.2% for surotomycin at 125 mg and 250 mg twice daily, respectively; while 35.6% for vancomycin given as 125 mg four times per day (29). From a microbiological perspective, it is tempting to speculate that surotomycin's bactericidal activity and narrower spectrum than vancomycin contributes to reducing recurrence. However, the in vitro findings of this study do not provide a direct explanation for the superiority of surotomycin compared to vancomycin in reducing recurrence.

The present study reported herein provides a solid framework from which to rationalize several recent findings on the in vitro activities against *C. difficile*. Indeed, reported observations that surotomycin is bactericidal against logarithmic cultures with a long post-antibiotic effect, reduces toxin and spore production and has a low propensity to select for *de novo* resistance in *C. difficile* (19, 28), can be rationalized as being consistent with membrane as the biological target.

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386 **TABLES AND FIGURES**

387

388 **Table 1**: In vitro activities of surotomycin and other first-line drugs for the treatment of

389 CDI

	*Activity µg/mL								
	BAA-1875 (078)			R20291 (027)			IT0843 (001)		
	MIC	MBC_{LOG}	MBC_{STA}	MIC	MBC_{LOG}	MBC_{STA}	MIC	MBC_{LOG}	MBC_{STA}
Surotomycin	0.25	4	2	1	8	16	0.125	8	16
Metronidazole	0.5	2	8	0.5	8	16	0.25	1	16
Vancomycin	0.5	>128	>128	2	>128	>128	2	>128	>128

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391 *MBC_{LOG}, minimum bactericidal concentration against logarithmic cells; MBC_{STA}

392 minimum bactericidal concentration against stationary phase cells.

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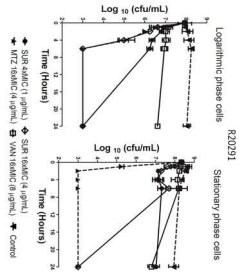
404 **Figure 1**: Time-kill kinetics of antibiotics against logarithmic-phase (left) and stationary-
405 phase (right) BAA-1875 cultures. Various concentrations of SUR = surotomycin; MTZ =
406 metronidazole; and VAN = vancomycin are shown in the legend.
407
408 **Figure 2**: Time-kill kinetics of antibiotics against logarithmic-phase (left) and stationary-
409 phase (right) R20291 cultures. Various concentrations of SUR = surotomycin; MTZ =
410 metronidazole; and VAN = vancomycin are shown in the legend.
411
412 **Figure 3**: Dissipation of the membrane potential and effects on membrane permeability
413 of *C. difficile,* shown as histogram half overlays. Representative data from three
414 independent cultures of BAA-1875 is shown, following exposure to drugs at 16×MIC. **A.**
415 CCCP was used as a control for dissipation of membrane potential and in **B** nisin is used
416 as a pore forming control; vancomycin is a negative control. CON = Control, SUR =
417 Surotomycin (4 µg/mL), DAP = Daptomycin (16 µg/mL), CCCP = Carbonyl cyanide m-
418 chlorophenyl hydrazine (2 µg/mL), NSN = Nisin (8 µg/mL), VAN = Vancomycin (8
419 µg/mL). Filters for FITC = Fluorescein Isothiocyanate, PI = Propidium Iodide.
420
421 **Figure 4:** Effects of inhibitory and bactericidal concentrations of surotomycin (SUR) on
422 macromolecular biosynthesis in *C. difficile*. In **A**, effects against BAA-1875 is shown for
423 SUR (0.25 and 4 µg/mL); whereas in **B** effects against R20291 is shown for SUR (1 and
424 16 µg/mL). Control drugs are also shown in both **A** (Gatifloxacin = 64 µg/mL; Rifaximin
425 = 0.96 µg/mL; Fusidic Acid = 2 µg/mL; Ampicillin = 8 µg/mL) and **B** (Gatifloxacin =
426 512 µg/mL; Rifaximin = 2 µg/mL; Fusidic Acid = 2 µg/mL; Ampicillin = 16 µg/mL).



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